

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Lal *et al.*

Art Unit: 1645

Application No. 09/763,397

Filed: February 16, 2001

For: RECOMBINANT MULTIVALENT MALARIAL
VACCINE AGAINST PLASMODIUM
FALCIPARUM

Examiner: Vanessa L. Ford

Date: June 4, 2002

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on June 4, 2002 as First Class Mail in an envelope addressed to: COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.

June 11, 2002
Tanya M. Harding, Ph.D.
Attorney for Applicant

COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.131

COPY OF PAPERS
ORIGINALLY FILED

I, Ya Ping Shi, hereby declare as follows:

1. I am a co-inventor of the subject matter described and claimed by the patent application referenced above, *i.e.*, United States application No. 09/763,397 (hereafter the '397 application). I currently am employed by the Centers for Disease Control and Prevention (CDC), the assignee of the '397 application, which is located in Atlanta, Georgia. I was employed by the CDC while developing the invention described and claimed in the referenced application.

2. I understand that claims pending in the present application have been rejected in view of Gilbert *et al.*, *Nature Biotechnology*, 15: 1280-1284, 1997. I understand that Gilbert *et al.*, has been cited as allegedly anticipating certain claims pending in the referenced application, or, in the alternative, as allegedly rendering the claimed embodiments obvious.

3. The publication date of Gilbert *et al.*, is November 1997. United States Provisional Application No. 60/097,703 was filed on August 21, 1998. However, the co-inventors named on the '397 application invented the subject matter covered by the claims pending in the '397 application well prior to the November 1997 date that Gilbert *et al.*, became available as a reference.

4. Accompanying this Declaration as Exhibit A are photocopies of pages from my laboratory research notebook. These copies are true and accurate facsimile copies of photocopies

of the corresponding pages from my laboratory notebooks. All dates stated on these pages have been redacted.

5. All entries on the notebook pages of Exhibit A were made prior to November 1997.

6. The ideas and concepts demonstrated by Exhibit A arose from work conducted for the CDC in Atlanta, GA. These ideas and concepts are embodied in the claims of the '397 application. Thus, conception and reduction to practice of the invention recited in the claims of the '397 application, as discussed in more detail below, occurred in the United States of America prior to November 1997.

7. Exhibit A consists of 21 pages of laboratory notebook pages. Exhibit B consists of one page of CDC Biotechnology Core Facility Records. The contents of these pages of Exhibits A and B, and pertinent statements made on these pages are discussed below.

A. Exhibit B is a record from the CDC Biotechnology Core Facility showing the dates of my request for oligonucleotide synthesis, and the sequences of the requested oligonucleotides. These requests were made prior to November 1997. These oligonucleotides were used as is depicted in Figure 2 of the specification to amplify the synthetic vaccine antigen gene construct using Polymerase Chain Reactions (PCRs). The oligonucleotides of Exhibit B consist of both forward and reverse complementary sequences of SEQ ID NO: 1 of the application, with overlapping sequences acting as primers for the amplification in either the forward or reverse direction.

B. Pages 1-6 of Exhibit A display the planning strategy for the PCR synthesis of the synthetic gene construct. Set forth are relevant calculations for PCR reactions and primers used to generate quantities of the synthetic gene construct. Also shown are electrophoresis gels used to visually confirm the size of PCR-generated products.

1) Page 1 shows the calculation and strategy for serial PCRs. As is set forth at the top of page one, "AA" was my short hand for the PCR reaction involving oligonucleotides G0, GL, G1, and G2 of Exhibit B. "BB" was my short hand for the PCR reaction involving oligonucleotides G3-G6, and "CC" was my short hand for the PCR reaction involving oligonucleotides G7-G12.

2) Pages 2-5 show several experiments, ending with success as indicated by my comment "works well" on page 5. Reactions DD-II as depicted were successive rounds of PCR that joined the amplified fragments into the final synthetic gene construct.

C. Page 6 shows an electrophoresis gel on which I ran and visualized four samples at different concentrations from PCR reactions (the central four bands of the gel). Next to the gel is my comment "good!" indicating that the size of the band corresponding to the PCR product in each sample appeared to be the correct size.

D. Pages 7-10 shows that the PCR product was isolated and purified from the electrophoresis gel shown on page 6. Next, the purified product was cut with restriction endonucleases with BamH1 and NotI (shown as steps #3 and #4 in Figure 2) for cloning into the expression vector pBluescript. The resulting sequence was SEQ ID NO: 1 of the application. Also shown on page 8 are the ligation reaction conditions for the ligation reaction, followed by restriction endonuclease reactions to evaluate the success of the ligation. The vector containing the fragment was then transformed into cells and plated onto agar plates. Positive clones were identified by their white color, indicating that the blue color-producing gene characteristic of a vector without a cloned segment had been interrupted with a cloned fragment. Page 10 sets forth the conditions for the PCR reaction to confirm that the correct gene fragment had been ligated into the vector (*i.e.*, to identify positive clones). My notation indicates that seventeen positive clones (numbers 1-4, 6, 8, 17, 21, 22, 25-27, 31, 33, 36, 39 and 40) were identified.

E. Page 11 shows an electrophoresis gel displaying samples of the PCR products.

F. Page 12 shows a single and double digesting experiment to confirm that the cloned fragment was properly oriented and was of the correct size. Clones 3, 26, and 33 were discarded by this experiment, leaving fourteen correct clones.

G. Page 13 shows the methods for the transformation of two plasmids, pBacPAK8 and pBacPAK9 with the synthetic gene construct for expression in Baculovirus. Also shown is an electrophoresis gel displaying samples of digested and undigested plasmid.

H. Page 14 shows another electrophoresis gel containing samples of DNA that were purified and digested with restriction endonucleases NotI and BamH1, to confirm that the cloning into the Baculovirus expression vectors had been successful. My notation "orders are no problem" indicates that the clones were correctly oriented, and my statement "confirm 11, 20, 63 clones are true clones" indicates that I considered these clones to be successful. The depicted gel

shows the results of restriction endonuclease digestion showing the two bands of each clone (lanes 4, 5 and 8, respectively). I identified clone number 20 as the clone that would be sequenced to confirm correctness at the molecular level. The sequencing indicated that clone 20 contained a single mutation. Therefore, I sequenced a second clone, number 63. The sequencing indicated that clone 63 also contained a single mutation. In comparing the location of the mutations in clones 20 and 63, I found the mutations were located in different segments. Thus, I subsequently performed experiments to generate a subclone that would contain the correct segments of clones 20 and 63.

I. Page 15 sets forth conditions for methylation experiments, which were run to protect restriction endonuclease sites in the vector. Following these reactions, the correct segments of clones 20 and 63 (as shown on the bottom of pg. 15) were excised and ligated into the Baculovirus expression plasmid pBacPAK8.

J. Page 16 shows a basic diagram of the recombinant vaccine antigen gene as cloned into the Baculovirus expression vectors. As shown, the construct contains portions of both clones 20 and 63.

K. Page 17 shows a gel wherein the products of the second ligation reaction of the correct segments of constructs 20 and 63 into the pBacPAK8 expression vector were run to confirm the size of the construct.

L. Page 18 shows the success of my ligation experiment, as confirmed by visualization of the bands on the electrophoresis gel. My statement that "clones 21, 31, 33, 35 are positive" indicates that the ligation reaction was successful.

M. Page 19 shows an electrophoresis gel displaying the results of BamH1 restriction endonuclease digestion, to confirm the successful clones.

N. Page 20 shows an electrophoresis gel displaying the results of BamH1 and NotI restriction endonuclease digestion. My notation "save clone 31A and 31B" indicates that these clones were successful. I subsequently confirmed these results by sequencing clones 31A and 31B.

O. Page 21 contains my notation "miniprep for sending product to Hassian (sic)." This refers to co-inventor Dr. Seyed Hasnain, who tested the expression of the synthetic gene construct in the Baculovirus expression system.

8. All statements made herein and of my own knowledge are true and all statements made on information are believed to be true. Furthermore, these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

06/05/2002

Date

Ya Ping Shi

Name

Ya Ping Shi

EXHIBIT A

First PCR

AA: G7C - G2	50.0	94°C 5min	8 cycle (p139)
BB: G3 - G6	50.0	94°C 45"	
CC: G7 - G12	50.0	45°C 1min	
		72°C 1.5min	

Strong/weak

AA:	2 x 4 = 8ul	73.5
BB:	2 x 4 = 8ul	65.5
CC:	2 x 6 = 12ul	61.5

16ul dNTP
10ul Buffer
0.5ul Taq

26.5ul

Second PCR

53.5

16ul dNTP
10ul Buffer
0.5ul Taq

5ul oligo 1
5ul oligo 2

46.5

94°C 5min

94°C 45"

45°C 1min

72°C 1.5min

25 cycle
(p141)

AA	DD1	1ul	52.5 + 5 + 5	
	DD2	2.5ul	51 + 5 + 5	G0
	DD3	5ul	48.5 + 5 + 5	G2
	DD4	10ul	43.5 + 5 + 5	
BB	EE1	1ul	52.5 + 5	
	EE2	2.5ul	51 + 5	G3
	EE3	5ul	48.5 + 5	G6
	EE4	10ul	43.5 + 5	

C	FF1	1ul	52.5 + 5	
	FF2	2.5ul	51 + 5	G7
	FF3	5ul	48.5 + 5	G12
	FF4	10ul	43.5 + 5	

Redo CC₀: G7 - G12 = 12ul

dNTP	16ul	c.; Taq
10x Buffer	10ul	
H ₂ O	61.5ul	
	<u>100ul</u>	

94°C 5min
 94°C 45"
 40°C 1min
 72°C 2min

} 8 cycle

CC₁ G7 - G8 (only do second PCR) = 4ul + 69.5ul^{H₂O}
 CC₂ G9 - G12 2x4 = 8ul + H₂O 65.5

Do SOE G₀ - G₆

	DD ₁ + EE ₁	H ₂ O	16ul dNTP
G ₀ G ₁	1ul + 1ul = 2ul	61.5	100ul Buffer
G ₁ G ₂	2.5ul + 2.1ul = 5ul	58.5	5ul G ₀
G ₂ G ₃	5ul + 5ul = 10ul	53.5	5ul G ₁
G ₃ G ₄	10ul + 10ul = 20ul	43.4	0.5 Taq
			<u>36.5</u>

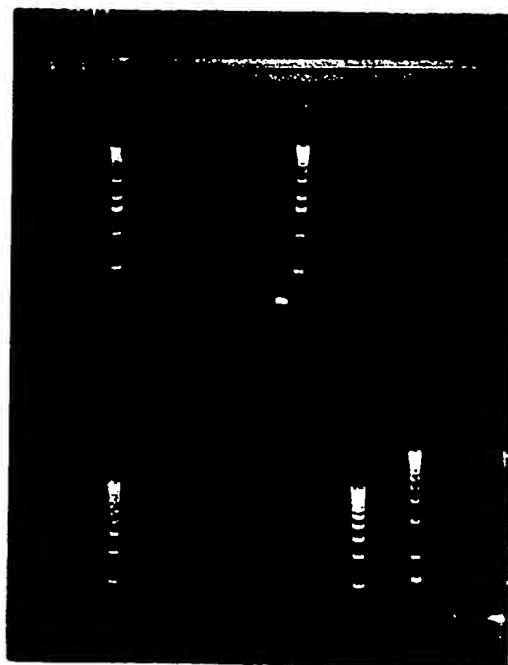
program 141

FF ₁	} CC ₁	primers	67.5	H ₂ O	16ul dNTP
FF ₂		G7	1ul	62.5	10ul Buffer
FF ₃		G12	2.5ul	61	oligos 10ul
FF ₄			5ul	58.5	Taq 0.5
FF ₅	} CC ₂	primers	1ul		
FF ₆		G9	2.5ul		94°C 5min
FF ₇		G12	5ul		94°C 45"
FF ₈			10ul		40°C 1min
				72°C 2min	8 cycles

2

#41

Result GG-4



FF-3 did not work probably because oligo ?
prepare new temp oligo GG - G12 also AG-1065

Redo: $CC'_2 \rightarrow CC''_2$ and CC''_3 .

23.5

CC''_2 G9 G10 G11 G12 $\times 2 = 8ul$. 65.5

CC''_3 G9 G10 G11 $AL1064 \times 2 = 8ul$. 65.5

\times
works
well

16ul dNTP.

10ul Buffer.

0.5 Tag

Same to before.

Second PCR.

FF''₁
FF''₂
FF''₃
FF''₄ } CC''_2 primers
G9
G12

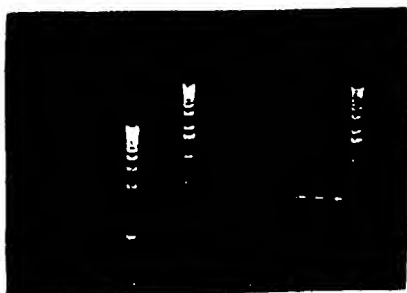
1-20
1ul 62.5
2.5ul 61
5ul 58.5
10ul 53.5

16ul dNTP
10ul buffer
0.1ug 10ul
Tag 0.5ul.

FF''₅
FF''₆
FF''₇
FF''₈ } CC''_3 G9
AL1064
works well

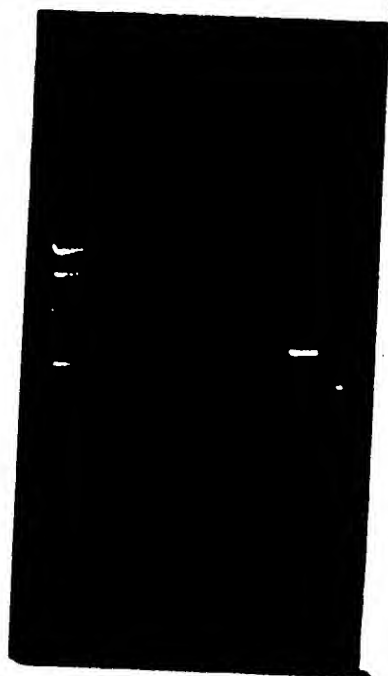
Same to before.

1141





114



11



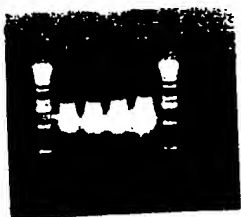
Set for G₁₇ - G₁₁ + AL-1065

	CC ₁	FF ₅	H ₂ O	63.5	16ml dNTP
HH ₁	1ul	+ 1ul	61.5		10ul buffer
HH ₂	2.5ul	+ 2.5ul	58.5		5ul G ₇
HH ₃	5ul	+ 5ul	53.5		5ul AL-1065
HH ₄	10ul	+ 10ul	43.5		0.5 T ₉₉
					<u>36.5</u>

program #41

	G ₇	+ HH ₁	H ₂ O	63.5	16ml dNTP
II ₁	1ul	+ 1ul	61.5		10ul buffer
II ₂	2.5ul	+ 2.5ul	58.5		5ul AL-1064
II ₃	5ul	+ 5ul	53.5		5ul AL-1065
II ₄	10ul	+ 10ul	43.5		0.5 T ₉₉
					<u>36.5</u>

program #41



good!

Further cleaning and cloning,
sequencing.

A: Run gel and cut and clean.

① gene clean (from product of PCR)

② gel clean through column (according introduction of manufacturer) (50ul of PCR product tube one is pellet (store in -20°C)

another ~~is~~ has 20ul water. From this, 10ul was take for digestion.

B: digestion:

Not 1 :
26ul water
3ul Buffer
1ul NOT I

1h 37°C

II, Gene clean

II, column clean

pellet.

BamHI

26ul H₂O
3ul buffer
1ul BamHI

1h 37°C

Ligation

Water 13ul

Vector 1ul (BamHI and NOT I digest)

5x lig buffer 4ul

T4 ligase 2ul

Control I

Control II

15ul

"

"

"

"

over night 4°C

NotI digestion:

Vector:

10ul
3ul
3ul
4ul
10ul

Vector (concent 3.2ug/ul)

10x buffer

BSA

NotI

H₂O

30ul 37°C 1.5h

target

II₂ and control (MSP-1)

2ul H₂O

3ul BSA

3ul 10x Buffer

2ul Enzyme

30ul 37°C 1.5h

NotI BamHI digestion

Vector

BamHI

4ul

Buffer

3ul

water

23

30ul

37°C 1.5h

Target

2ul

Buffer

3ul

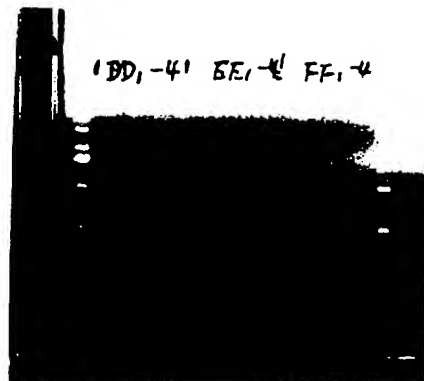
water

25ul

30ul

37°C 1.5h

Result



1DD, -41 BE, -4 FF, -4

FF, -4 did not work because first PCR (CC) annealing temp was too high

Need redo CC (first PCR), then FF, -FF, -FF, -FF

ligation as before
transformation as before

result. not so much white clones. probably vector
was not properly digested.
clone further purify vector.

pick up 40 clone grow overnight.

cell PCR: as regular. 10ul cell 94°C 5min.

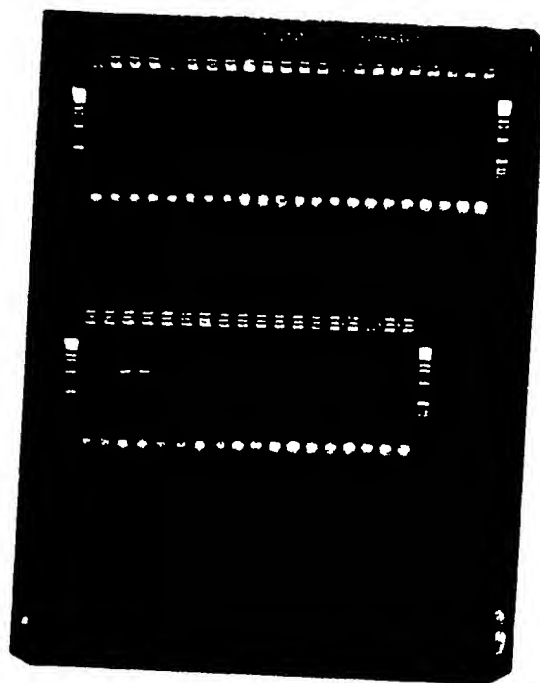
eligo	AL1064	2.5ul
	AB1065	2.5ul
	Buffer	5ul
	dNTP	8ul
	Taq	0.5
	1+20	27.75
		<hr/> 40ul

15 cycle 94°C 45" 50°C 45" 72°C 2m

print the clone

1, 2, 3, 4, 6, 8, 17, 21, 22,
25, 26, 27, 31, 33, 36, 39, 40,

for back

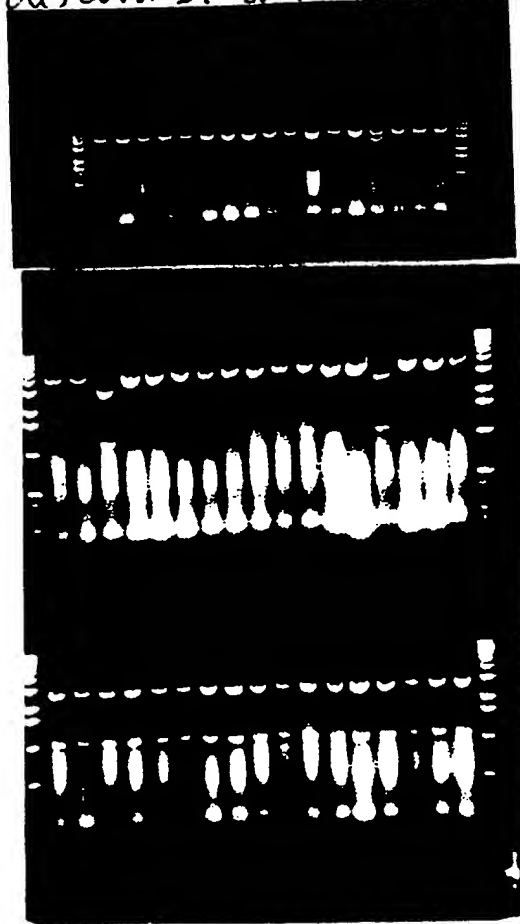


Save cell
Savolas SGV R# Synthetic Gene Vaccine
Eaton to sign

digest all positive (17) clones (based on PCR)

Single digestion: BamHI or NotI
double digestion BamHI and NotI.

Result: Clone 3, 26, 33 are not pure clones.
discard or don't use them



2, 6

Plasmid pBacPAK8 and pBacPAK9 (from Sanyo)
21g/100ul 21g/100ul

Transformation:

10ul plasmide (200ng)

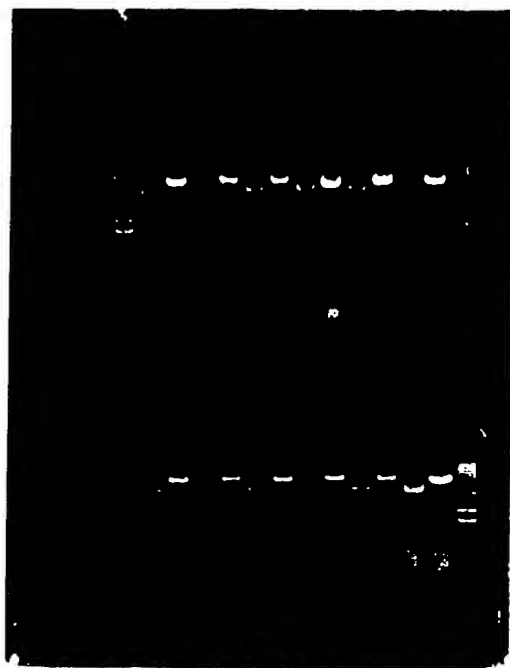
100ul XL-blue cell

procedure as regular.

plating overnight
growth well

Miniprep of pBacPAK8 and pBacPAK9 -

run undigested and digested plasmid

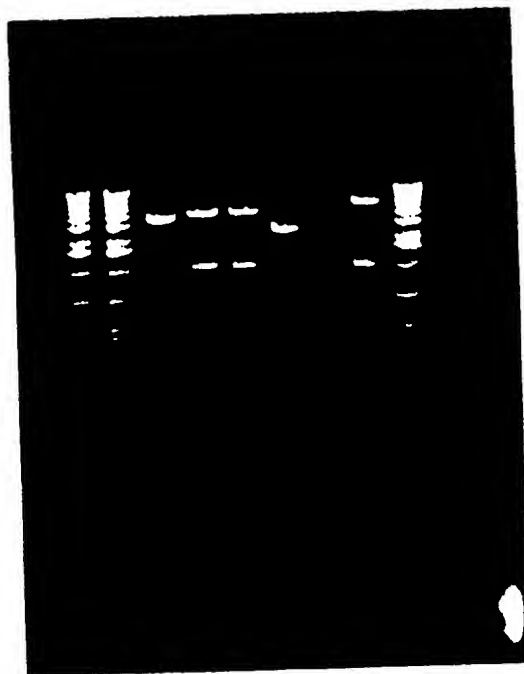


$100ng/\mu l \times 19 =$

1.9ul.

13.1g

This result confirm that. ~~no~~ orders are
no problem. do confirm (11) (20) (63) clones
are true clones:



Will sequence clone 20.

Methylation:

Clone 63 Vector correct.

Clone 20 Most target correct.

Clone 63 methylation.

Reaction:	3ul	Tagi methylation
	3ul	NEB 4 Buffer
	0.3ul	BSA
	22.2ul	H ₂ O
	1.5ul	Mix SAM
		1 hr 65°C

Mix: 50ul NEB 4 Buffer + 450ul H₂O + 1.25ul SAM

0.6ul Nac. (SM)

60ul Ethanol (100%)

Hind II cut

Clone 63 (two pieces very big)

Clone 20 (more pieces
vector small)

run gel

Standard

20

63

Standard

(more small / + a: big)

15

reaction conditions:

3 ul	buffer
6 ul	NotI
21 ul	1420

1.5 hr 37°C

Result:

clone 20

clone 63

|||||

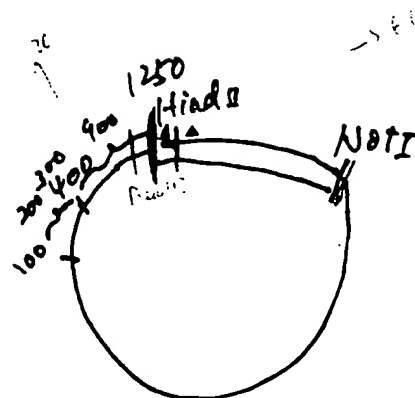
—

Δ

Δ 1 kb
— 0.9 kb

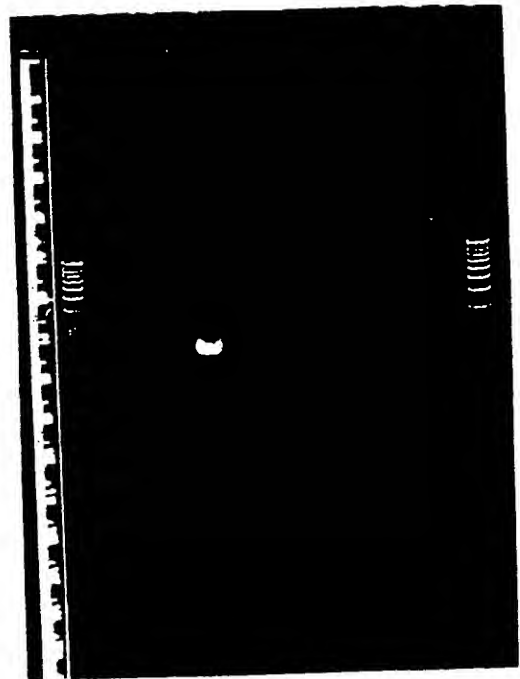
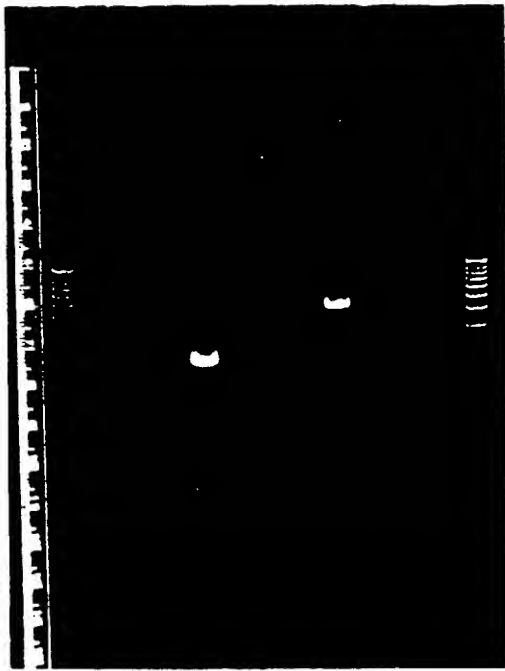
— — 1 kb

— 0.8 kb



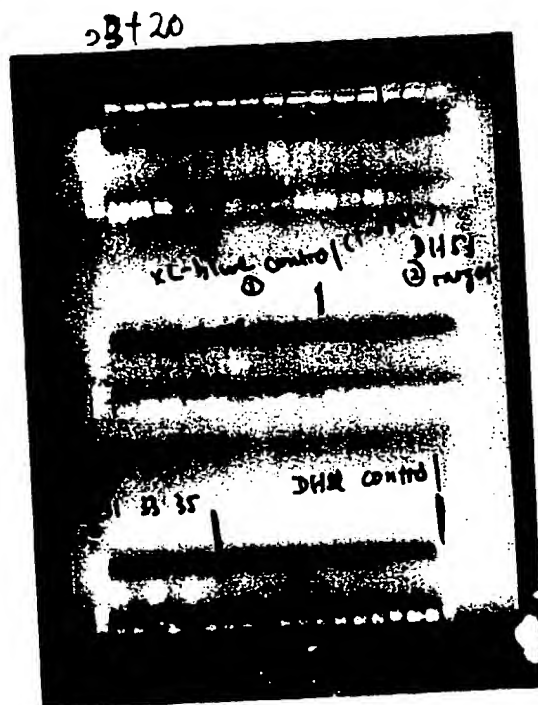
Δ cut fragment

ligation: as routine



clone 63 + 20 ligation see before

clone PCR primer: AL1097
AL1064



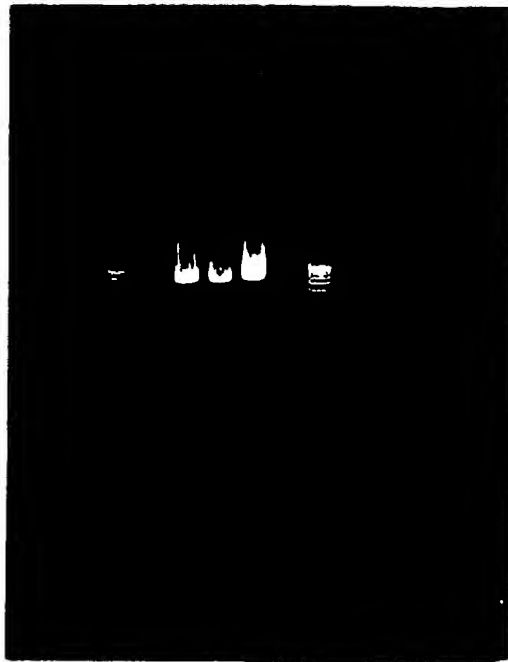
clones 21, 31, 33, 35
are positive

save as name:

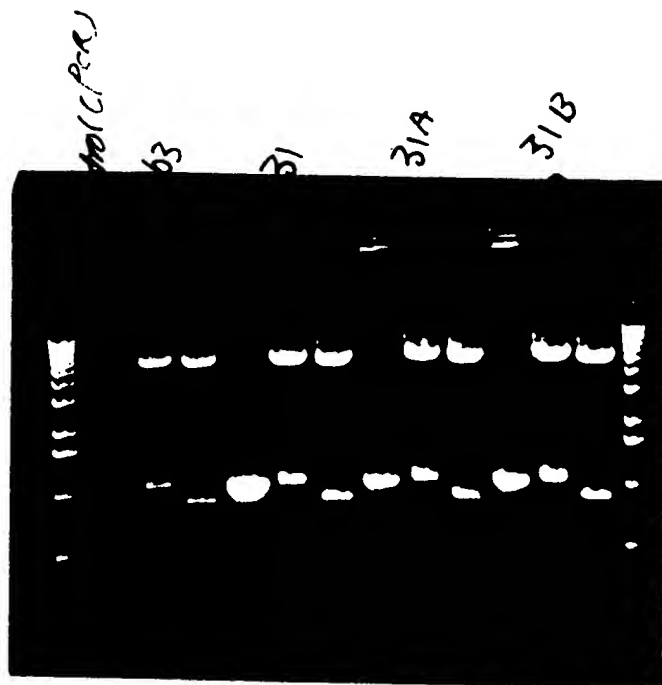
~~380TS/CL-DT21/63+20~~

name
Pac8/63+20/number

Banc HT digestion: 63+20 (21, 31, 33, 35) 6!



original promoter methylation



Save clone 31A and 31B
named as pac8/63+20/31A and 31B

Date	Oligo ID	DNA Syn No.	Sequence	Length-mer
	G0	953882	GTGGGATCCATGAAATTCCTAGTCAACGTTGCCCTTGT TTATGGTCGTGTACATTTCTTACATCTATGCGGcalcatca tcalcatcat	94
	GL	953883	ttatctcatcttaccctcalgatgatgatgatgat	40
	G1	953884	gaaggtaaagatgaagataaaAGAGATGGAATTAACGAAGACMAC GAGAAATTAAAGGAACCAAAACATAAAAAATTAAAGCAAC CAGGGGATGGTAAATCcttggctcccatgtagtla	120
	G2	953885	tattacgacattaacacacTGAACATTTTCCATTTTACAAATTT TTTTTCAATATCATTTTCAATATCTAATTCGTCCTTAGGTT TTCACACAGTtaccatcatggygaccaag	120
	G3	953886	TGTGTTTAATGTCGTAATAGTAATTCGGATGTTTCAGA CATTTAGATGAAGAGAGAAGAAATGTAATGTTTATTAGAAG ATTGAGGTAGCAACCGAAAGAAATCAGATGTGAATGTAC	120
	G4	953887	tggtatcttcaaatCCATCAGGATTTGCATTTGGGTTGCCG TTGGGTTTGCATTTGAAATTGTCAATTTGCACAATAGGCTT AGAATCAGGTTTAgIaccatcacatglattht	120
	G5	953888	gaaittgaagatataccacATGTAATGAATTTTCAGCAATTGAT CTTGGAATGCTGAAATAATATGATAAATGATGAACCAC AACATTTATGGGAaatcaicacacccatggaag	120
	G6	953889	gcattgttaagaigtgCTTAGTTCAAGTTATACCTATAAATTTAG AATTTGCTTTTATATACTGATACAACCTTTTATCATTTGGTT TATATAATcttctaalgagtagtlat	120
	G7	953890	aacacattcaaaacaatgctGGACAACATGGACATATGCATGTA ACGAGAGGGAAGATGAGAGAAGCGCTTACTAAGGAATATG AAGATATTTGTTTTGAaagagttacatalatgata	120
	G8	953891	ctthaattttcataicGTTAAATGTTGTCATATTGTTTAGGTTGA TCACTTTTTTGATATGGATGTTCCCAATAATTCTGTCCTCT TCCAAAGTTtalcatalatglaaacctt	120
	G9	953892	agattatgaanaaaitaaagAAGGTAAGCCCTTGATAAATTTGGA AATATCTATGATTATCACATATGAGCAATTCTAGTCCATCTAG TACAAAGTCATCAagltccatcaaatglaaaic	120
	G10	953893	alccttalatttcgctaaaacCTTTTCAATAAATTATACCGAAGAA ATCTCTGATTTTCAGCTTTAAATTTTTCATTAAATCTTGTAG CTAGACTagcigtatttaccatttgaic	120
	G11	953894	tttagcgaatalaagaTGAATTAGAGTTAGTATCAGGAGATA TGTTGCAAGAATCAAAATAGATATACTATTTTCTTAttgaa taggcgcccgcgac	104
	G12	953895	gtcgcgcccgcctattcaaa	20